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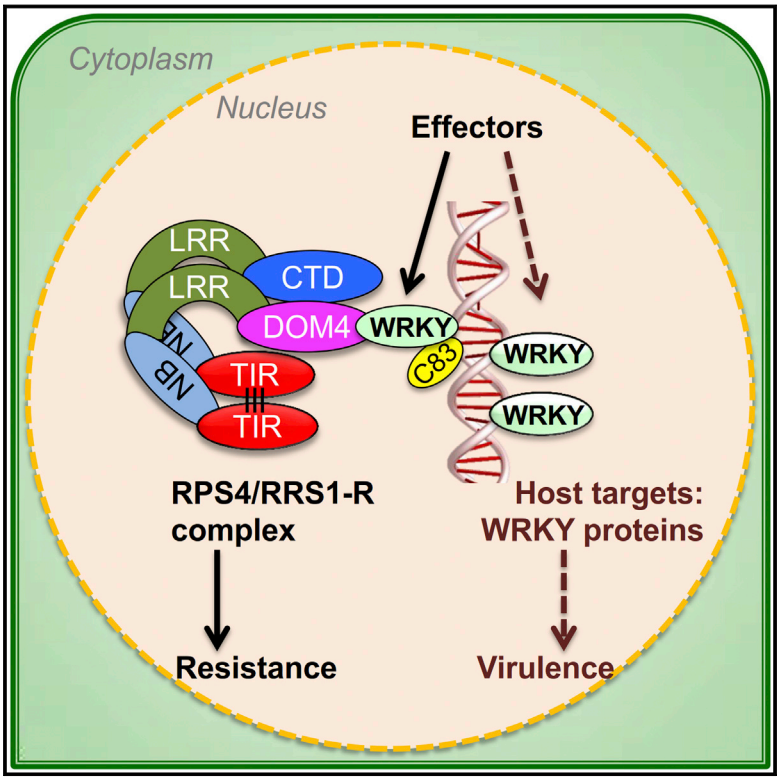
A Plant Immune Receptor Detects Pathogen Effectors that Target WRKY Transcription Factors

Graphical Abstract



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In Brief

An Arabidopsis intracellular immune receptor complex, one component of which contains A WRKY DNA binding domain, detects specific bacterial effectors to activate defense. Acetylation of WRKY domains by an effector likely interferes with host defense and this domain in the receptor complex enables detection of pathogen effectors that broadly target WRKY domain proteins.

Highlights

- A plant immune receptor detects two different bacterial effectors via a WRKY domain
- One pathogen effector PopP2 acetylates lysines and another, AvrRps4 binds the WRKY domain
- This regulation occurs for other plant WRKY proteins involved in immunity
- Receptor WRKY domain enables detection of effectors that target such proteins

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A Plant Immune Receptor Detects Pathogen Effectors that Target WRKY Transcription Factors

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SUMMARY

Defense against pathogens in multicellular eukaryotes depends on intracellular immune receptors, yet surveillance by these receptors is poorly understood. Several plant nucleotide-binding, leucine-rich repeat (NB-LRR) immune receptors carry fusions with other protein domains. The *Arabidopsis* RRS1-R NB-LRR protein carries a C-terminal WRKY DNA binding domain and forms a receptor complex with RPS4, another NB-LRR protein. This complex detects the bacterial effectors AvrRps4 or PopP2 and then activates defense. Both bacterial proteins interact with the RRS1 WRKY domain, and PopP2 acetylates lysines to block DNA binding. PopP2 and AvrRps4 interact with other WRKY domain-containing proteins, suggesting these effectors interfere with WRKY transcription factor-dependent defense, and RPS4/RRS1 has integrated a "decoy" domain that enables detection of effectors that target WRKY proteins. We propose that NB-LRR receptor pairs, one member of which carries an additional protein domain, enable perception of pathogen effectors whose function is to target that domain.

INTRODUCTION

To ward off disease, animals have evolved cell-autonomous innate immunity, and chordates have also evolved an adaptive immune system. Plants rely solely on innate immunity, activated by cell surface or intracellular receptors (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Although most eukaryotes resist most potential pathogens, how recognition of pathogen molecules activates defense is poorly understood.

Plant pathogens deliver diverse virulence factors ("effectors") to suppress host defense. Intracellular receptors recognize effectors either directly or indirectly and usually comprise nucleotide binding-leucine rich repeat (NB-LRR) receptor Resistance (R) proteins, which resemble mammalian Nod-like Receptors (NLRs) (Dangl and Jones, 2001; Dodds and Rathjen, 2010; Jones and Dangl, 2006). NB-LRR-mediated effector-triggered immunity (ETI) often leads to a *h*ypersensitive cell death *r*esponse (HR). Recognized effectors that trigger ETI can be called avirulence (Avr) proteins. *Arabidopsis thaliana* has ~120 genes encoding full-length NB-LRRs, while most mammals have ~20 NLRs (Jacob et al., 2013). Mammalian NLRs can recognize pathogen molecules such as flagellin or peptidoglycan (Eitas and Dangl, 2010; Kofoed and Vance, 2011; Maekawa et al., 2011; von Moltke et al., 2013). NB-LRR and NLR signaling requires their *s*ignal *t*ransduction *A*TPase with *n*umerous *d*omains (STAND) domains (Leipe et al., 2004). The NB domain of plant NB-LRRs is of the nucleotide-binding, Apaf-1, R-protein, and CED-4 (NB-ARC) class (van der Biezen and Jones, 1998a; Williams et al., 2011) and is proposed to be ADP-bound in the auto-inhibited or "off" state and ATP bound in the activated or "on" state (Maekawa et al., 2011; Williams et al., 2011). Plant NB-LRRs usually carry a Toll/Interleukin-1 receptor/Resistance protein (TIR) or a coiled-coil (CC) motif in their amino termini. The two plant NB-LRR subfamilies have distinct genetic requirements and phylogenetically distinct NB domains (Jones and Dangl, 2006; McHale et al., 2006). In mammalian NLRs, the amino-terminus may carry diverse other domains (Maekawa et al., 2011; von Moltke et al., 2013).

Activation of ETI by NB-LRRs can be initiated via direct interaction of an effector with a NB-LRR protein, ("the ligand-receptor model") (Catanzariti et al., 2010; Dodds et al., 2006; Jia et al., 2000; Krasileva et al., 2010; Ravensdale et al., 2012). Alternatively, NB-LRR proteins can "guard" host proteins that are targets of effectors (guard model) (Dangl and Jones, 2001; van der Biezen and Jones, 1998b) or host proteins that have evolved to resemble those targets (decoy model) (van der Hoorn and Kamoun, 2008). The Pyrin inflammasome senses bacterial effector-dependent modification of Rho GTPases (Xu et al., 2014) suggesting the guard/guardee model is also relevant to mammalian systems (Keestra et al., 2013; Stuart et al., 2013).

Plant and animal NLRs can function in pairs (César et al., 2013; Eitas and Dangl, 2010; Narusaka et al., 2009; Williams et al., 2014). In mouse, recognition and response to either bacterial flagellin or PrgJ requires ligand-dependent interactions of the NLR NLRC4 with either NAIP5 or NAIP1/NAIP2, respectively (Kofoed and Vance, 2011; Tenthorey et al., 2014). The *Arabidopsis* TIR-NB-LRRs *RPP2A* and *RPP2B* are both required for

downy mildew resistance (Sinapidou et al., 2004), and rice CC-NB-LRRs RGA4 and RGA5 are both required for race-specific resistance to rice blast fungus. In NB-LRR pairs, one or both NB-LRR proteins usually carry fusions with other protein domains (Eitas and Dangl, 2010; Meyers et al., 2003). For example, in RGA4/RGA5, which confers recognition of two fungal effectors, AVR-Pia and AVR1-CO39, RGA5 carries a C-terminal RATX domain by which it interacts with the effectors (Césari et al., 2013, Césari et al., 2014).

The *Arabidopsis* Resistance to *Pseudomonas syringae* 4 (*RPS4*) and Resistance to *Ralstonia solanacearum* 1 (*RRS1*) genes encode TIR-NB-LRR proteins and are both required for race-specific resistance to bacteria and to a fungus (Deslandes et al., 2003; Gassmann et al., 1999; Narusaka et al., 2009; Williams et al., 2014). *RRS1* in *Arabidopsis* accessions Nd-1 and Ws-2 (*RRS1-R*) confers recognition (with RPS4) of type III effector (T3E) PopP2, an acetyltransferase of the YopJ family (Tasset et al., 2010) and of *P. syringae* pv. *pisi* T3E AvrRps4 (PstDC3000-AvrRps4) (Gassmann et al., 1999). The Col-0 allele of *RRS1* (*RRS1-S*) confers AvrRps4, but not PopP2, recognition. RPS4 and RRS1-R (but not RRS1-S) also confer resistance to the fungus *Colletotrichum higginsianum* (Birker et al., 2009; Narusaka et al., 2009). RRS1 and RPS4 proteins interact in part via their TIR domains; this interaction is essential for defense activation (Williams et al., 2014).

How the RPS4/RRS1 complex perceives effectors is unknown. PopP2 and RRS1 interact in split-ubiquitin yeast-2-hybrid (Y2H) (Deslandes et al., 2003) but the interacting domain of RRS1 was not defined. PopP2 interacts with both RRS1-R and RRS1-S, resulting in nuclear localization of RRS1, so RRS1-S interaction with PopP2 per se is insufficient for defense activation (Deslandes et al., 2003). AvrRps4 recognition by the RPS4/RRS1 pair is also not understood, though RRS1 co-immunoprecipitates (coIPs) with AvrRps4 as it does with PopP2 (Williams et al., 2014). AvrRps4 is cleaved in planta releasing a C-terminal anti-parallel coiled-coil fragment ($AvrRps4^{C}$) containing residues Gly134-Gln221 (Sohn et al., 2012). Mutation of the Lys135-Arg-Val-Tyr138 (KRVY)-motif to four alanine residues, or Glu187 to alanine, abolishes AvrRps4-triggered HR and immunity (Sohn et al., 2009, 2012).

Two other *Arabidopsis* TIR-NB-LRR proteins also carry WRKY domains (Narusaka et al., 2009). WRKY proteins are transcription factors harboring a conserved WRKY domain, which contains the WRKYGQK motif followed by a $C_{x4-5}C_{x22-23}H_xH$ or $C_{x7}C_{x23}H_xC$ zinc-finger motif (Chi et al., 2013). In *Arabidopsis*, most WRKY genes are implicated in defense (Chi et al., 2013; Dong et al., 2003).

We report here that both PopP2 and AvrRps4 interact with the RRS1 WRKY domain. PopP2 acetylates RRS1-R and RRS1-S WRKY domains at both lysines of the canonical WRKYGQK sequence. Acetylation of RRS1-S by PopP2 abolishes its capacity to recognize AvrRps4. Acetyl-lysine mimic substitution alleles of RRS1-R, but not RRS1-S, trigger effector-independent RPS4-dependent defense activation. We found that AvrRps4 and PopP2 interact with other *Arabidopsis* WRKY proteins, and other WRKY proteins are also acetylated by PopP2. We infer that the RPS4/RRS1 complex enables plants to detect effectors that interfere with WRKY protein function in plant defense. The discovery of multiple effectors that target WRKY proteins, and the evolution of immune receptors that detect such effectors, emphasizes the significance of WRKY proteins for plant immunity. Our key finding is thus that plant NB-LRR receptor pairs have evolved in which one member carries protein domains that enable perception of the action of pathogen effectors that target that domain, while the other activates defense upon such perception.

RESULTS

Wild-Type and Mutant AvrRps4 and PopP2 Interact with RRS1-S and RRS1-R in the Plant Cell Nucleus

PopP2 associates with RRS1-S and RRS1-R and they co-localize in the nucleus (Tasset et al., 2010; Williams et al., 2014). AvrRps4 also co-immunoprecipitates (coIPs) with RRS1 and RRS1/RPS4 (Williams et al., 2014). We examined the subcellular location of these interactions. Bimolecular fluorescence complementation (BiFC) confirmed nuclear association of PopP2-cCFP, or the non-recognized enzymatically inactive mutant PopP2-C321A-cCFP, with RRS1-R-nCerulean and RRS1-S-nCerulean when transiently co-expressed with RPS4-HA in *Nicotiana benthamiana* (*Nb*) (Figures 1 and S1A). All fusion proteins are driven by the CaMV 35S promoter (35S). We validated PopP2/RRS1 and PopP2-C321A/RRS1 interactions using coIP assays (Figure S1B). RPS4/PopP2 or RPS4/PopP2-C321A does not coIP (Figure S1C). The BiFC signals for RRS1-R with PopP2 or PopP2-C321A appear as "speckles," while the signals for RRS1-S with either PopP2 or PopP2-C321A are more evenly dispersed (Figure 1). BiFC also shows nuclear association of AvrRps4-cCFP and the non-recognized mutants AvrRps4-KRVYAAAA-cCFP or AvrRps4-E187A-cCFP (Sohn et al., 2012), with both RRS1-S-nCerulean and RRS1-R-nCerulean (Figure 1). AvrRps4 interactions with the RPS4/RRS1 complex and with RRS1 alone were validated by coIPs (Figures S1D and S1E).

PopP2 Interacts with and Acetylates Lysines in the WRKY Domain of RRS1-S and RRS1-R Proteins

PopP2 enzymatic activity is essential for defense activation via RRS1-R (Tasset et al., 2010). We tested if RPS4/RRS1-R-mediated resistance to PopP2 involves acetylation of RRS1-R protein by PopP2, using an anti-acetyl-lysine antibody (α-Ac-K) (Tasset et al., 2010). We co-expressed 35S:PopP2-GFP or 35S:PopP2-C321A-GFP, with 35S:RPS4-HA and 35S:RRS1-R or 35S:RRS1-S fused to a tandem 6xHis and 3xFLAG epitope tag (HF) in *Nb* leaves by agroinfiltration. GFP, AvrRps4-GFP, and PopP2-C321A-GFP were used as negative controls. After protein extraction and immunoprecipitation (IP) with FLAG beads, immunoblot analysis with the α-Ac-K antibody revealed acetylation of RRS1-R and RRS1-S in the presence of PopP2, but not GFP, AvrRps4 or PopP2-C321A-GFP (Figures 2A and S2A). The RRS1 acetylation signal was stronger in the absence of RPS4 or with TIR domain hetero-dimerization mutants RRS1-R SH-AA co-expressed with RPS4 SH-AA (Williams et al., 2014) (Figure 2A). This suggests proper assembly of RRS1 protein into an RPS4/RRS1 complex results in fewer available sites of RRS1 for acetylation by PopP2. PopP2, but not PopP2-C321A, auto-acetylates (Figures S2B, S2C, S3A, and S4E).

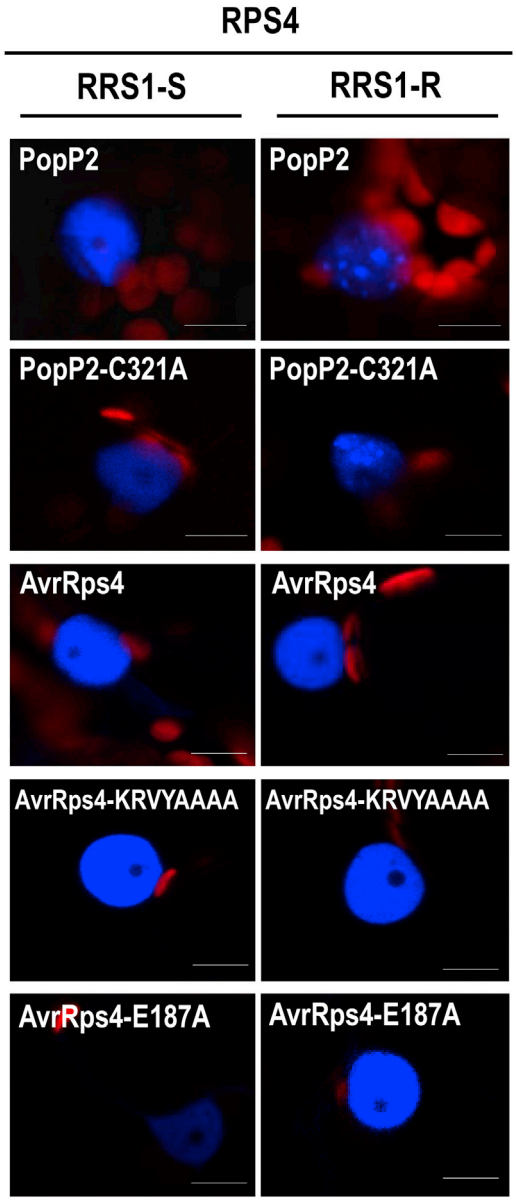

Figure 1. PopP2, AvrRps4, and Their Mutants Interact with RRS1-R and RRS1-S in the Nucleus

BiFC assays reveal close proximity of RRS1-R or RRS1-S with PopP2 and AvrRps4 effectors and PopP2-C321A, AvrRps4-KRVYAAAA, and AvrRps4-E187A mutants, after agro-infiltration of *Nb* leaves. RRS1 alleles were C-terminally tagged with nCerulean, and effectors were C-terminally tagged with cCFP. The interaction of the two tags creates CFP (blue signal) if two proteins are adjacent. Images were recorded at 48 hpi. White scale bar, 15 μm. See also Figure S1.

Using mass spectrometry (MS), we identified RRS1 peptides acetylated by PopP2 (Figures S2D–S2I). Several acetylated peptides were detected, including mono- and di-acetylated peptides that show an ~42 Da mass shift on lysine (K) residues in the WRKY domain (Figures 2D and S2D–S2I). We transiently expressed HF-tagged RRS1-R and RRS1-S in *Nb*, digested affinity-purified protein, and used selected reaction monitoring (SRM) to quantify the relative level of acetylation of these peptides in the presence of PopP2, PopP2-C321A or GFP (Figures 2B, 2C, and S2J–S2M; Table S1). This method allowed relative quantification of acetylated peptides between samples by normalizing to protein levels using several non-modified control peptides from the protein of interest (Table S1). We identified four PopP2-dependent acetylated lysines, in and nearby the WRKY domains of both RRS1-R and RRS1-S, corresponding to acetylation of K1217, K1221, K1247, and K1276 in RRS1-R (Figures 2B–2D and S2J–S2M).

Only full-length RRS1 protein has been reported to interact with PopP2 (Deslandes et al., 2003). We replaced the WRKY domain of both RRS1-R and RRS1-S with the bacterial LexA DNA-binding domain (Figure S2N) (Fogh et al., 1994). LexA contains a DNA binding domain with no known target in plant DNA. PopP2 failed to coIP with RRS1-R WRKY/LexA or RRS1-S WRKY/LexA (Figure S2O), suggesting that the WRKY domain is necessary for interaction of RRS1 with PopP2.

Acetylation of RRS1-S and RRS1-R by PopP2 Leads to Reduced Binding to W-Box DNA Sequences

Two conserved lysine residues (K^1 and K^2) in the WR**K**^{1}YGQ**K**^{2} region of the WRKY-domain interact directly with DNA, and mutations of these conserved lysines disrupt protein-DNA interaction (Duan et al., 2007; Maeo et al., 2001). Furthermore, the RRS1-R mutant allele that was first reported as "sensitive to low humidity-1" (*slh1*), contains a leucine insertion in its WRKY domain, shows reduced W-box DNA binding and confers constitutive activation of defense (Noutoshi et al., 2005). We tested if acetylation of the WRKY domain by PopP2 affects DNA binding by RRS1. To this end, we performed electrophoretic mobility shift assays (EMSA), testing the ability of both RRS1-R and RRS1-S to bind W-box DNA in the presence of PopP2 or PopP2-C321A (Figures 3A, 3B and S3A). For the EMSA assays, we transiently co-expressed RRS1-R or RRS1-S and RPS4 in the presence of PopP2 or PopP2-C321A mutant in *Nb*. In previous studies, bacterial expression of an RRS1 fragment containing the WRKY domain only (exons 6 and 7, Figure S2N) was used for DNA binding assays (Noutoshi et al., 2005). We investigated RRS1 DNA-binding using full-length RRS1 in the presence of RPS4. The target DNA was a 29-bp double-stranded oligonucleotide containing three W-boxes (W-box DNA probe). Furthermore, a mutant form of the 29 bp double-stranded oligonucleotide was used as a negative control (Figure S3B). Both RRS1-R and RRS1-S WRKY domains bind radiolabeled W-box DNA; this binding can be competed with unlabeled W-box DNA (Figures 3A, 3B and S3B). The interaction of both RRS1 alleles with W-box DNA was inhibited in the presence of PopP2 but not in the presence of PopP2-C321A or GFP (Figures 3A, 3B, and S3A). These results indicate that the acetyl-transferase activity of PopP2 is required to inhibit RRS1 DNA-binding. RRS1-R and RRS1-S LexA replacement and *slh1* alleles do not bind W-box DNA (Figure 3C). These data also indicate that PopP2 targets the RRS1 WRKY-domain; we infer that acetylation of lysines in the WRKYGQK domain reduces the affinity of RRS1 and of the RPS4/RRS1 complex for W-box DNA sequences.

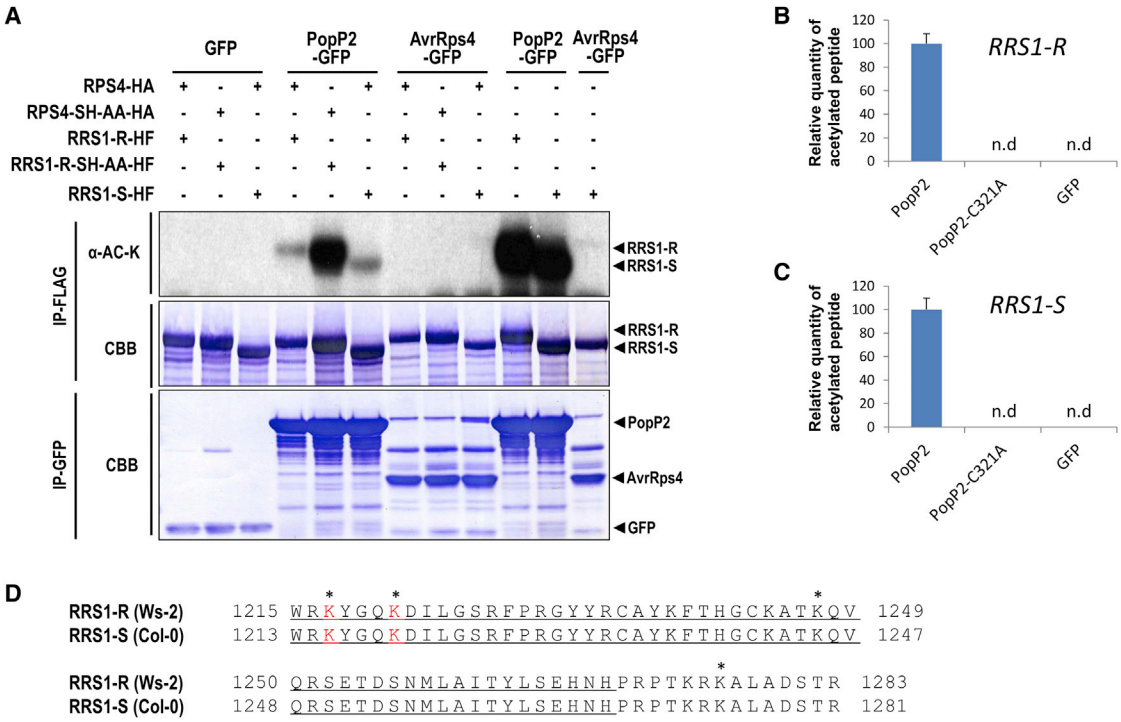

Figure 2. PopP2 Acetylates the WRKY Domain of RRS1-R and RRS1-S Proteins

(A) Immunoblots analyzed with anti-acetyl-lysine antibody (α-Ac-K) show lysine (K) acetylation of RRS1-R, RRS1-S, and RRS1-R-SH-AA by PopP2-GFP in the presence or absence of RPS4 or RPS4-SH-AA. GFP and AvrRps4-GFP are negative controls. RRS1 proteins tagged C-terminally with HF (6xHis 3xFLAG) were IPed from *Nb* leaf extracts. Equal amounts of FLAG-purified RRS1 proteins were stained with Coomassie brilliant blue (CBB). Equal amounts of GFP-IPed PopP2-GFP, AvrRps4-GFP and GFP were stained with CBB.

(B and C) Relative levels of the **K**YGQ**K**DILGSR peptide in the WR**K**YGQ**K** motif of RRS1-R (B) and RRS1-S (C). Relative levels of the double-lysine-acetylated KYGQKDILGSR 2+ peptide, determined by SRM, shown as an average (n = 4), with bars showing SE; *n.d.*, not detected.

(D) Acetylated lysines in and around the RRS1 WRKY domain (underlined) indicated by asterisks. Lysines found in (B) and (C) histograms are highlighted in red. See also Figure S2 and Table S1.

PopP2 Acetylation of the RRS1-S WRKY Domain Inhibits AvrRps4 Recognition

We tested if acetylation of RRS1-S WRKY domain by PopP2 compromises recognition of AvrRps4. In agroinfiltration assays in *Nicotiana tabacum* (*Nt*), leaf sections co-expressing 35S:RRS1-S and 35S:RPS4 show HR in response to AvrRps4, but not to PopP2, recapitulating the recognition specificity in *Arabidopsis* Col-0 (Figures S4A and S4B). This AvrRps4-triggered RPS4/RRS1-S-dependent HR is suppressed by co-expression with PopP2, but not with the PopP2-C321A mutant (Figure 4A). PopP2-C321A interaction with RRS1 protein (Figures 1 and S1B) indicates that competition between AvrRps4 and PopP2 for RRS1 binding cannot explain the attenuation of AvrRps4 responsiveness by PopP2. We infer that the enzymatic function of PopP2 (acetylation of RRS1-S) likely causes the attenuation.

We next tested if PopP2 interferes with resistance to AvrRps4-carrying bacterial strains conferred by RPS4/RRS1-S in *Arabidopsis* Col-0. We used a *Pseudomonas fluorescens* strain Pf0-1 carrying a type III secretion system (Pf0-1:T3SS or Pf0-1/) (Thomas et al., 2009) that delivers PopP2, or a PopP2-C321A mutant. Pf0-1/PopP2, but not Pf0-1/PopP2-C321A, restores growth of *P. syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) carrying AvrRps4, to a level comparable to *Pst*DC3000 + *Pf0*-1/PopP2 (Figure 4B). Therefore, resistance in *Arabidopsis* Col-0 against *Pst*DC3000/AvrRps4 is reduced by PopP2-dependent acetylation of RRS1-S. PopP2-C321A interacts with RRS1-S in the nucleus (Figure 1) but does not suppress AvrRps4 recognition. We infer that PopP2 acetylation of the RRS1-S WRKY-domain causes loss of AvrRps4 binding and loss of recognition of AvrRps4 by the RPS4/RRS1-S complex.

We also tested the HR suppression ability of PopP2 on the RPP1^{WsB}/ATR1 system by agroinfiltration in *Nt*. PopP2 does not suppress the HR triggered by the recognition of ATR1 by RPP1^{WsB} (Figure S4C), suggesting that PopP2-mediated suppression of AvrRps4 recognition by RPS4/RRS1-S is not due to general HR-suppressing activity of PopP2.

We tested if AvrRps4 coIPs with RRS1 exons 6 and 7 (E67), which encode the WRKY domain. In RRS1-R and RRS1-S, the WRKY domain (60 aa) is identical, while RRS1-R E67 (E67-R) carries an 83 aa C-terminal extension compared to E67-S. AvrRps4 coIPs with E67-R or E67-S when co-expressed in *Nb* (Figure 4C). E67-R association with AvrRps4 is greatly reduced in the presence of PopP2, but not PopP2-C321A (Figure 4C). E67-R coIPs equally strongly with PopP2 and PopP2-C321A

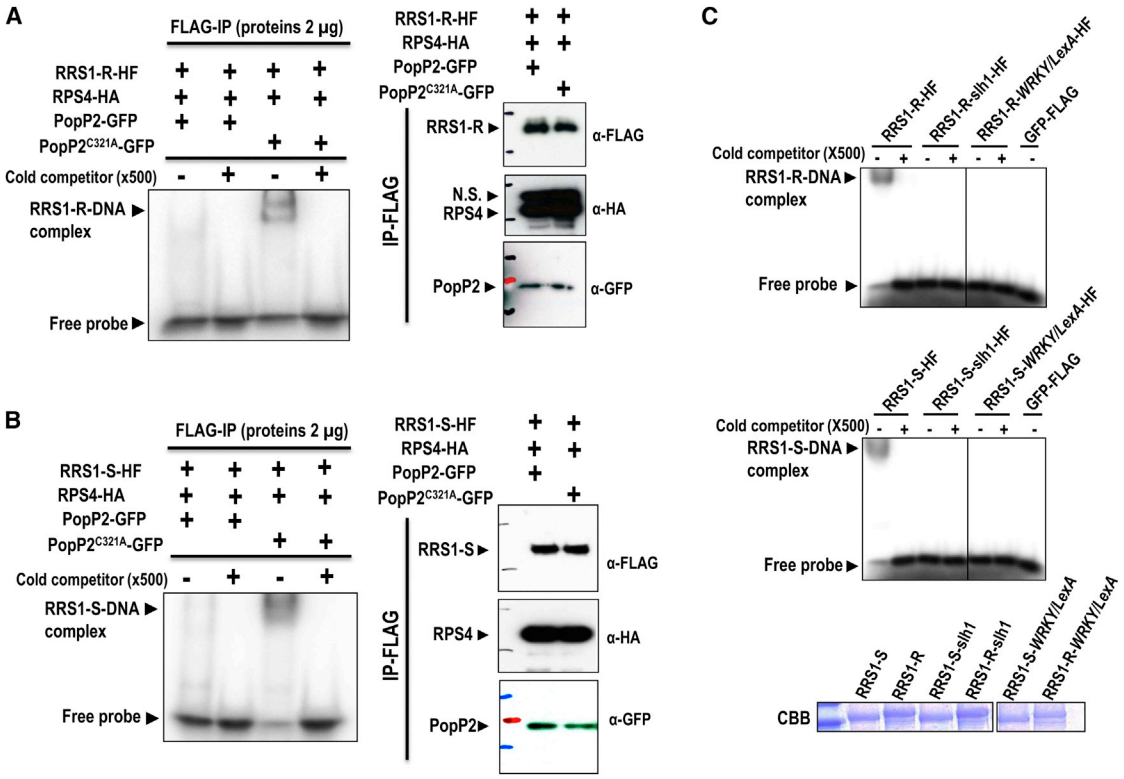

Figure 3. PopP2 Acetylation Reduces RRS1 Affinity for Synthetic *W*-Box DNA

(A and B) Electrophoretic mobility shift assays (EMSAs) showing DNA-binding of RPS4/RRS1 in the presence of PopP2 or PopP2-C321A. EMSAs were performed with RRS1-R (A) or RRS1-S (B), co-expressed with RPS4, and either PopP2 or PopP2-C321A. RRS1 protein was IPed with anti (α)-FLAG antibody, and 2 µg of purified protein was incubated with ^{32}P-labeled synthetic 3x*W*-box double stranded DNA (dsDNA) oligonucleotide for 1 hr on ice. Complexes were electrophoresed in a 4% native acrylamide gel and signals detected by an FLA-5000 image analyzer (Fujifilm). For the competition assay of RRS1-S and RRS1-R proteins, RRS1 proteins were co-incubated with ^{32}P-labeled synthetic *W*-box dsDNA mixed with 500-fold excess of cold *W*-box dsDNA competitor. Control immunoblots were performed using the same coIP samples (right panels in A and B).

(C) EMSA showing absence of DNA-binding of RRS1-R-WRKY/LexA and RRS1-S-WRKY/LexA mutant proteins, in which the WRKY domain of RRS1 was replaced by a LexA domain and of RRS1-R-slh1 or RRS1-S-slh1 mutant proteins. GFP-FLAG is a negative control. These experiments were repeated three times with identical results. Equal loading was confirmed by SDS-PAGE and Coomassie staining.

See also Figure S3.

(Figure S4D), indicating that PopP2-mediated interference is not due to competition for binding. These data suggest that the WRKY domain of RRS1 interacts with AvrRps4. Using the acetylation-specific antibody, we detected acetylation of E67-R by PopP2 but not mutant PopP2-C321A (Figure S4E). PopP2 specifically acetylates two lysine residues (K^1 and K^2) in the RRS1 WRK^1YGQK^2 domain, and this acetylation blocks AvrRps4 binding, implying that AvrRps4 directly interacts with the WRKY domain of RRS1.

Recognition of AvrRps4 by RPS4/RRS1 Requires a Functional RRS1 WRKY Domain

We constructed RRS1-R and RRS1-S WRKYGQK mutants, either WRKYGQ**R** or WR**R**YGQK in which R cannot be acetylated, or WR**Q**YGQK or WRKYGQ**Q**, in which Q is a mimic of acetylated lysine and tested AvrRps4 recognition by these mutants.

We co-expressed RRS1-R carrying mutations in WRKYGQK together with RPS4 and with PopP2 or AvrRps4 in *Nt* leaves. RRS1-R WRKYGQK domain mutants to WRKYGQ**R** but not WR**R**YGQK lose recognition of both PopP2 and AvrRps4 (Figure 5A). This indicates a crucial and specific role for K^2 in the WRK^1YGQK^2 motif (K1221 in RRS1-R) in recognition of both effectors. RRS1-S acetyl-lysine mimic mutants to WR**Q**YGQK, WRKYGQ**Q**, or double WR**Q**YGQ**Q,** fail to recognize AvrRps4, suggesting the K^1 contribution to AvrRps4 recognition is disrupted by a Q but not an R substitution (Figure 5B). These observations suggest that recognition of AvrRps4 via the RRS1 WRKY domain is sensitive to subtle changes in the K^2 position of the WRK^1YGQK^2 motif in RRS1-R and RRS1-S and explains why acetylation of K^1 and K^2 in RRS1-S WRKYGQK suppresses recognition of AvrRps4.

The RRS1-R^{SLH1} allele confers RPS4-dependent HR after transient expression in *Nt* (Sohn et al., 2014). Notably, RRS1-S^{SLH1} shows no such autoimmune phenotype when co-expressed with RPS4 (Figure S5A). Moreover, like RRS1-S WRKY-acetylation by PopP2, the leucine insertion in RRS1-S^{SLH1} abolishes AvrRps4 responsiveness (Figure S5A).

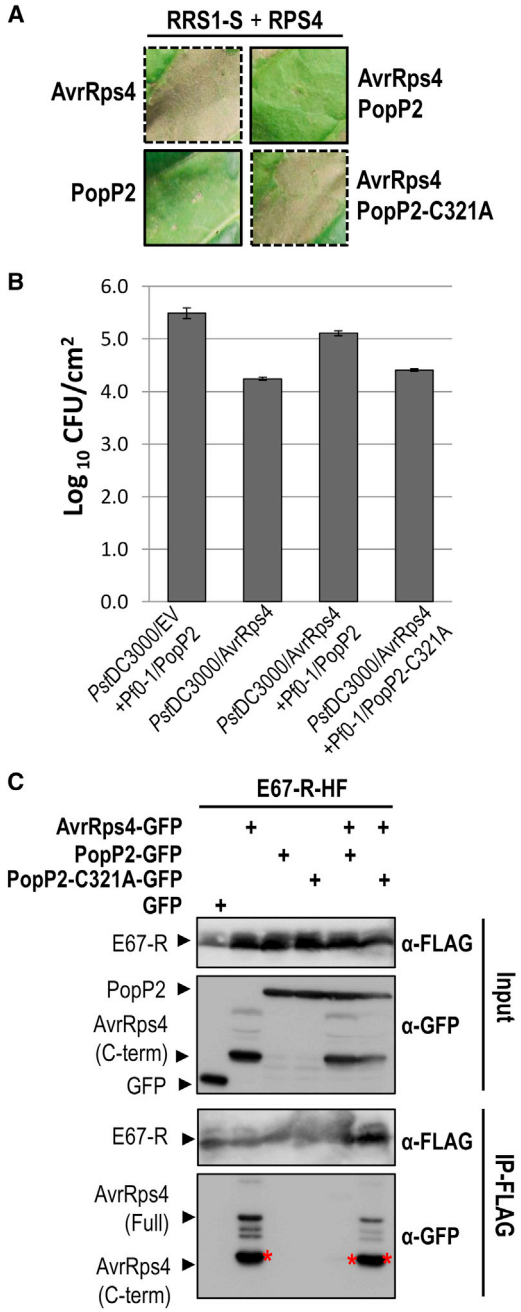

Figure 4. PopP2 Acetylation of the RRS1-S WRKY-Domain Inhibits AvrRps4 Recognition and Binding

(A) Agroinfiltration assays in *Nt* leaves show that PopP2, but not PopP2-C321A, inhibits AvrRps4 recognition by the RPS4/RRS1-S complex. Each leaf section co-expresses RPS4, RRS1-S and either AvrRps4 or PopP2, or both AvrRps4 and either PopP2 or PopP2-C321A. Photographs assessing HR were taken 4 days post infiltration (dpi). This was repeated at least three times with similar results.

(B) Bacterial growth assays show PopP2, but not PopP2-C321A, inhibits resistance of *Arabidopsis* Col-0 to *Pst* DC3000 carrying AvrRps4 (shown as *Pst* DC3000/AvrRps4). Histogram shows growth of *Pst* DC3000 carrying empty vector (EV) or AvrRps4, measured 3 dpi in *Arabidopsis* leaves. *P. fluorescens* Pf0-1 carrying PopP2 or PopP2-C321A were co-infiltrated with *Pst* DC3000 strains. Means ± SD of three replicates per sample are given.

(C) PopP2-GFP, but not PopP2-C321A-GFP, inhibits AvrRps4-GFP association with E67-R-HF after transient co-expression in *Nb*. Immunoblots show the presence of proteins in total extracts (input) and after IP with α-FLAG or α-GFP beads (IP-FLAG or IP-GFP). Asterisks indicate the expected protein bands. See also Figure S4.

WRKY Domain Mutants that Lose Recognition of AvrRps4 Fail to Interact with AvrRps4

Although RRS1 E67 interacts with AvrRps4, E67 derivatives in which the RRS1 WRKY-domain was replaced with the bacterial LexA DNA binding domain (E67-S-WRKY/LexA and E67-R-WRKY/LexA) do not coIP AvrRps4 (Figure S5B). These results also indicate that the RRS1-WRKY domain is a binding domain for AvrRps4. However, full-length RRS1-R-WRKY/LexA or RRS1-S-WRKY/LexA still coIP with AvrRps4, suggesting additional RRS1 domains can interact with AvrRps4 (Figure S5C). AvrRps4 coIPs with RRS1-R exons 1–5 (E12345), which lacks the RRS1 amino acids C-terminal to the LRR domain including the WRKY domain and C-terminal extension, confirming that additional RRS1 domains in E12345 interact with AvrRps4 (Figure 5C).

We co-expressed wild-type or mutant E67-R or E67-S domains with AvrRps4 in *Nb* and tested for coIP. Both E67-R^{SLH1} and E67-S^{SLH1} fail to coIP with AvrRps4, suggesting a single leucine insertion in the WRKY domain abolishes affinity for AvrRps4 (Figure S5D). The RRS1-R^{K1221R} mutant does not recognize PopP2, indicating that acetylation of K^2 (K1221) is required for activation of PopP2-triggered and RRS1-R-mediated HR. The mutation of RRS1-R WRK^{1}YGQK^2 domain to WRKYGQ**R** but not to WR**R**YGQK loses recognition of both PopP2 and AvrRps4. Likewise, an RRS1-S acetyl-lysine mimic mutation to WR**Q**YGQK or WRKYGQ**Q** does not recognize AvrRps4. We co-expressed AvrRps4 with E67 forms of these mutant RRS1-R alleles and showed that AvrRps4 coIPs with E67-R but not with E67 WRKYGQ**R** or WRKYGQ**Q** mutants (Figures 5D and S5F).

To investigate the effect of AvrRps4 interaction with the RRS1 WRKY domain we performed EMSAs to test if RRS1-S and RRS1-R bind DNA in the presence of AvrRps4 (Figures S3C and S3D). Both RPS4/RRS1-S and RPS4/RRS1-R bind to radiolabeled W-box dsDNA. However, this interaction with W-box dsDNA was not significantly reduced by co-expression with AvrRps4.

In summary, the RRS1 WRKY domain interacts with AvrRps4, mutant forms of the WRKY domain that compromise responsiveness to AvrRps4 also compromise binding, but AvrRps4 binding to the WRKY domain does not reduce its affinity for W-box DNA.

RRS1 WRKY Interactions with AvrRps4 Are Necessary but Not Sufficient for Genetic Recognition of AvrRps4

E67-R interacts weakly with AvrRps4-E187A, but maintains strong affinity with AvrRps4-KRVYAAAA comparable to AvrRps4 (Figure S5E). We infer binding to the RRS1 WRKY domain is necessary but not sufficient for AvrRps4 recognition, and E187 is required for AvrRps4 interaction with the RRS1 WRKY domain. In contrast, the KRVY motif is irrelevant for WRKY domain binding, but is required for interacting with other components or domains required for recognition. Although AvrRps4 and

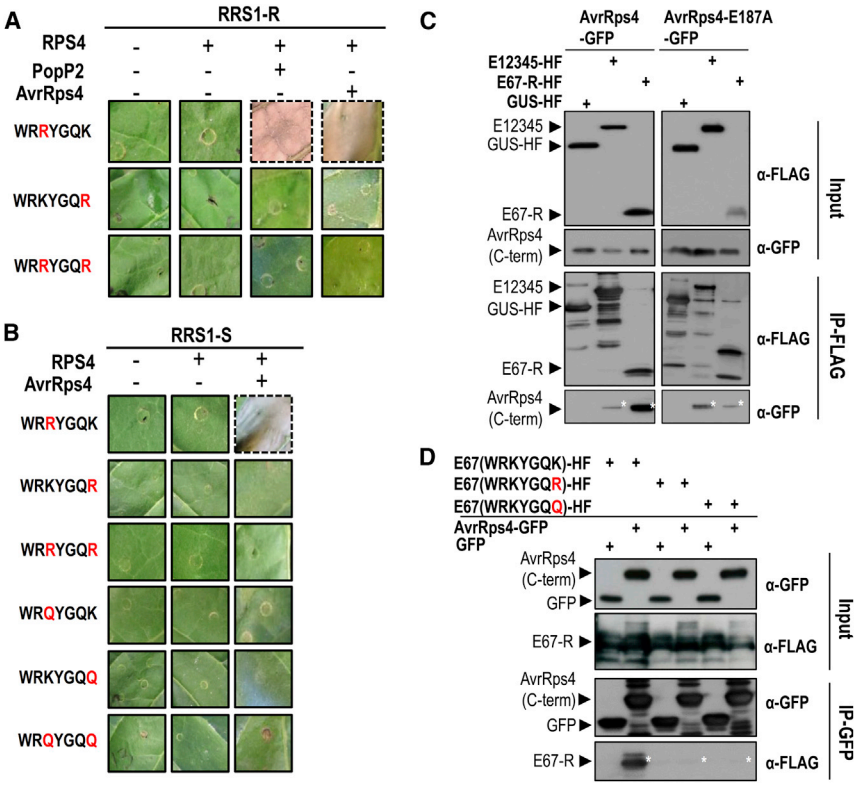

Figure 5. K1221 of RRS1-R (K^2 in WRK^1YGQK^2) Is Necessary for Recognition of PopP2 and AvrRps4

(A) Recognition of PopP2 and AvrRps4 in *Nt* leaves requires K1221 of RRS1-R. RRS1-R mutants (K1217R, K1221R and K1217R/K1221R) expressed alone or co-expressed with RPS4 and either PopP2 or AvrRps4.

(B) Acetyl-lysine mimic alleles (K to Q) of RRS1-S lose AvrRps4 recognition when co-expressed with RPS4. Each *Nt* leaf section was infiltrated to co-express RRS1-S mutants (K1215R, K1219R, K1215R/K1219R, K1215Q, K1219Q, and K1215Q/K1219Q) with or without RPS4 and AvrRps4. Photographs assessing HR were taken 4 days post infiltration (dpi). This was repeated at least three times with identical results. Leaf sections with HR are bordered by a dashed line.

(C) Association of AvrRps4-GFP or AvrRps4-E187A-GFP with protein encoded by exons 1–5 of RRS1 (E12345)-R-HF and E67-R-HF.

(D) AvrRps4-GFP associates with (E67)-R-HF, but not with E67-R(WRKYGQ**R**)-HF or E67-R(WRKYGQ**Q**)-HF mutants. Immunoblots show the presence of proteins in total extracts (input) and after IP with anti-FLAG beads (IP-FLAG). Asterisks indicate expected protein bands.

See also Figure S5.

AvrRps4-E187A differ in their interaction with the WRKY domain, they interact equally with RRS1 E12345 (Figure 5C). CoIP of AvrRps4-E187A with RRS1 E12345 could explain AvrRps4-E187A association with full-length RRS1 proteins in BiFC (Figure 1).

The C-Terminal Extension of RRS1-R Is Essential for PopP2-Dependent Defense Activation and RRS1-R^{SLH1} Auto-Immunity

The RRS1-R^{SLH1} allele causes effector-independent, RPS4-dependent, HR in *Nt* transient assays but RRS1-S^{SLH1} does not (Figure S5A). However, the DNA-binding capacity of both RRS1-R and RRS1-S is similarly disrupted by the *slh*1 mutation (Figure 3C) and by PopP2-dependent acetylation (Figures 3A and 3B). These results, together with loss of AvrRps4-triggered immunity due to acetyl-lysine substitutions in RRS1-S, suggest that PopP2 responsiveness by RRS1-R but not by RRS1-S, and the defense activation by RRS1-R^{SLH1} but not by RRS1-S^{SLH1}, involve the same mechanisms. To investigate which protein domains confer PopP2 responsiveness, we constructed protein chimeras between RRS1-R and RRS1-S by swapping the exon 7 of these two genes (Figure 6A). When introduced into RRS1-S, the 83 aa extension C-terminal to the WRKY-domain encoded by exon 7 of RRS1-R, enables RPS4-dependent PopP2 recognition and HR (Figure 6A). RRS1-R-E7S, lacking this C-terminal extension, does not confer RPS4-dependent PopP2 recognition, phenocopying RRS1-S (Figure 6A).

Acetyl-Lysine Mimic Alleles of RRS1-R, but Not RRS1-S, Show Effector-Independent, RPS4-Dependent HR

Acetylation of K1217 and K1221 by PopP2 disrupts DNA-binding and activates defense. RRS1-R (but not RRS1-S) acetyl-lysine mimic mutants to WR**Q**YGQK (RRS1-R^{K1217Q}) or WRKYGQ**Q** (RRS1-R^{K1221Q}) and the double mutant (WR**Q**YGQ**Q**) all trigger HR in the absence of an effector when expressed in *Nt* in combination with 35S:RPS4 (Figure 6B). Furthermore, the K1221Q mutation inhibits RRS1 binding to W-box DNA (Figure S3B). No HR was seen when RPS4 was co-expressed with RRS1-S^{K1215Q}, RRS1-S^{K1219Q}, and the double mutant RRS1-$S^{K1215Q/K1219Q}$ (Figure 5B).

We also tested the activity of lysine to arginine (K-to-R) mutations in RRS1 in *Nt* transient assays in the absence of effectors. When WR**R**YGQK, WRKYGQ**R** or WR**R**YGQ**R** mutations were introduced in RRS1-R or RRS1-S, no constitutive RPS4-dependent HR was seen after co-expression in *Nt* (Figures 5A and 5B).

Other WRKY-Domain Proteins Associate with AvrRps4 and PopP2 and Are Acetylated by PopP2

PopP2 and AvrRps4 interactions with the WRKY domain of RRS1 are required for activation of immunity. This suggests that RRS1 might detect effectors that interact with WRKY-domain proteins and impair their contribution to plant immunity. We tested if PopP2 and AvrRps4 associate with other WRKY-domain proteins. C-terminally GFP-tagged versions of both effectors were transiently co-expressed with several C-terminally HF-tagged WRKY proteins, selected because of their possible

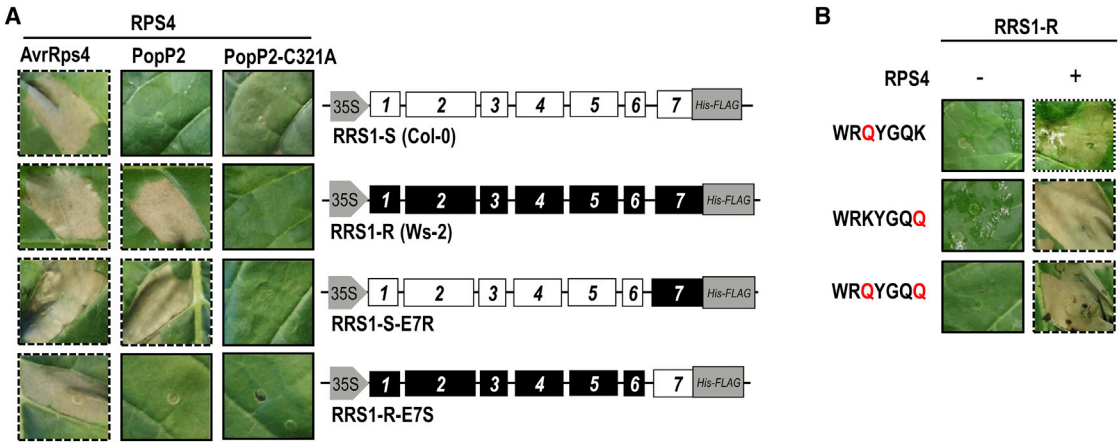

Figure 6. Recognition of PopP2 Is Determined by K1221 and the C-Terminal Extension of RRS1-R

(A) Assessing functionality of RRS1-R, RRS1-S and chimeric proteins in *Nt* leaves. RRS1-S-E7R consists of RRS1-S-E123456 fused to RRS1-R-E7R; RRS1-R-E7S consists of RRS1-R-E123456 fused to RRS1-R-E7.

(B) Acetyl-lysine mimic alleles of RRS1-R (but not RRS1-S; see Figure 5B) exhibit constitutive effector-independent RPS4-dependent HR in *Nt* leaves. Photographs assessing HR were taken 2 days post infiltration (dpi). Each experiment was repeated at least four times with identical results. Leaf sections with HR are bordered by a dashed line; weak HR is indicated by a dotted line.

involvement in plant defense. WRKY41, WRKY70, WRKY60, and WRKY33 coIP with PopP2-C321A (Figure 7A). PopP2-C321A was chosen because it interacts more strongly with RRS1 than does PopP2 (Williams et al., 2014). We analyzed coIP'd WRKY proteins by MS and detected acetylation of WRKY41, WRKY70, and WRKY33, but not WRKY60 by PopP2 at the conserved WRKYGQ**K** motif K that corresponds to K1221 in RRS1-R (Table S2). Furthermore, EMSA assays revealed that acetylation of WRKY41 by PopP2 reduces WRKY41 DNA binding ability (Figure 7B).

AvrRps4 coIPs with WRKY41, WRKY70, WRKY33, and WRKY60 (Figures 7C and S6). These results confirm that both PopP2 and AvrRps4 associate with some but not all WRKY-domain proteins. Acetylation of the WRKY-domain of any WRKY protein likely interferes with W-box DNA binding, and AvrRps4 binding may interfere with WRKY protein function by other means. We infer that the RPS4/RRS1 immune complex evolved to detect effectors that interfere with WRKY protein function.

DISCUSSION

Our data, and those in a companion paper by Le Roux et al. (2015), provide a simple and unifying hypothesis for how, and importantly, why, the RPS4/RRS1 complex recognizes PopP2 and AvrRps4. Multiple examples exist of direct recognition (Catanzariti et al., 2010; Dodds et al., 2006; Jia et al., 2000; Krasileva et al., 2010). However, some resistance proteins recognize effectors indirectly, via their effect on host targets (the "guard" model) (Dangl and Jones, 2001; van der Biezen and Jones, 1998b) or via recognition of "decoy" proteins that resemble effector targets and whose status is "guarded" by R proteins (van der Hoorn and Kamoun, 2008).

Both PopP2 and AvrRps4 interact with the WRKY domain of RRS1. Although AvrRps4 interacts with other domains of RRS1, PopP2 interacts specifically with the WRKY domain and acetylates lysines within the canonical WRKYGQK motif. Acetylation of these lysines in RRS1-S, which does not respond to PopP2, abolishes AvrRps4 recognition. The RRS1-R^{SLH1} allele (Noutoshi et al., 2005), with a leucine insertion in the WRKY domain, constitutively activates RPS4-dependent defense; we show here the RRS1-S^{SLH1} allele does not. RRS1-S^{SLH1} also cannot confer AvrRps4 recognition, and its WRKY domain does not bind AvrRps4. Mutating either K residue in the WRK^1YGQK^2 motif to Q, and mutating K^2 to R, abolishes AvrRps4 recognition and WRKY domain binding in RRS1-S. For RRS1-R, an acetyl-lysine mimic Q residue at the K^2 position in WRK^1YGQK^2 confers constitutive RPS4-dependent HR in *Nt*, similar to that conferred by the RRS1-R^{SLH1} allele. Importantly, we show that the K^2 in the WRK^1YGQK^2 motif is crucial for recognition of both PopP2 and AvrRps4. These data show unambiguously that the RPS4/RRS1-R complex recognizes both AvrRps4 and PopP2 via their interactions with the RRS1-R WRKY domain.

Do transient overexpression assays in *Nt* reflect mechanisms in *Arabidopsis*? In a mutant screen that recovered many mutations in RPS4 or RRS1, transient expression in *Nt* of mutant alleles recapitulated the loss of function in *Arabidopsis* (Sohn et al., 2014). Transient activation of RPS4/RRS1-dependent defense also activates tobacco defense genes as in *Arabidopsis* (Sohn et al., 2014). We did not test loss-of-function mutants in *Arabidopsis* because Ws-2 and Col-0 carry an additional locus, RPS4B/RRS1B, that confers AvrRps4 (but not PopP2) recognition (Saucet et al., 2015). Loss of function mutations in the TIR domains of RRS1 and RPS4 lose function in both *Arabidopsis* and tobacco (Williams et al., 2014). RPS4/RRS1 confer disease resistance in *Nicotiana* plants (Narusaka et al., 2013). Thus, tobacco transient assays likely recapitulate genetic requirements for defense activation by RPS4/RRS1.

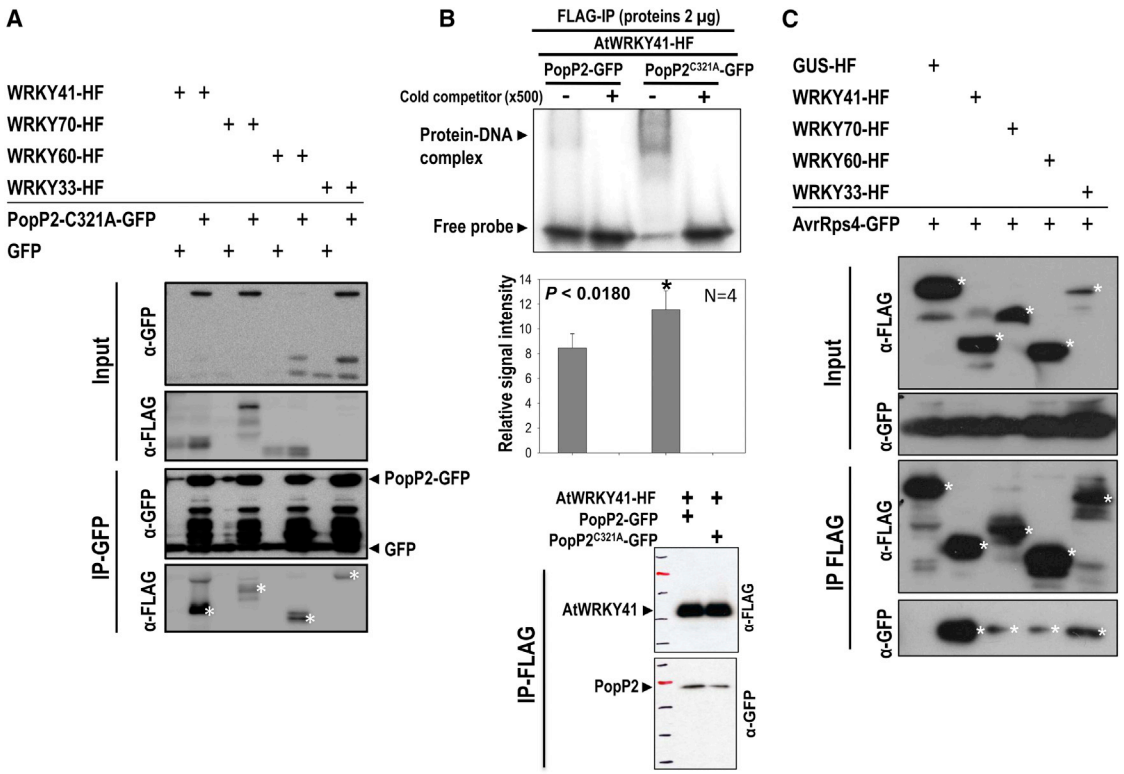

Figure 7. Other WRKY Proteins Associate with PopP2 and AvrRps4 and Are Acetylated by PopP2

(A) Tests of association between PopP2-C321A-GFP and WRKY41-HF, WRKY70-HF, WRKY60-HF, and WRKY33-HF. Immunoblots show the presence of proteins in total extracts (input) and after IP-GFP. GFP was used as a negative control.

(B) EMSAs were performed with α-FLAG-purified WRKY41 co-expressed with PopP2 or PopP2-C321A, as described in Figure 3 legend and in the Experimental Procedures. Control immunoblots were performed using the same coIP samples.

(C) CoIP assays testing association between AvrRps4-GFP and WRKY41-HF, WRKY70-HF, WRKY60-HF, and WRKY33-HF. Immunoblots show the presence of proteins in total extracts (input) and after IP-FLAG. GUS-HF was used as a negative control. Asterisks indicate expected protein bands. Each interaction was repeated at least twice.

See also Figure S6 and Table S2.

The discovery of *Arabidopsis* NB-LRR proteins that carry WRKY domains initially suggested such proteins reveal a direct link between detection of effectors (directly or indirectly) and activation of defense via WRKY transcription factors (Jacob et al., 2013). RPS4 has also been proposed to "guard" the plant defense regulator, EDS1, and interactions between EDS1 and AvrRps4 have been reported (Bhattacharjee et al., 2011; Heidrich et al., 2011). However, we favor the interpretation that EDS1 associates with TIR-NB-LRR proteins solely because EDS1 participates in TIR-NB-LRR signal transduction.

RRS1-S is acetylated by PopP2 in its WRKY motif, but loss of W-box DNA binding by RRS1-S is insufficient for defense activation. WRKYGQK acetylation must instead provoke intra- and inter-molecular reconfiguration within the RPS4/RRS1-R complex that also involves the C-terminal extension present in RRS1-R alleles, but not in RRS1-S alleles. This C-terminal extension is not required for AvrRps4 to activate the complex, but is required for PopP2 to activate the complex.

How NB-LRR proteins activate defense upon recognition is unknown (Jacob et al., 2013). We hypothesize RRS1 converts RPS4 into an activated state that resembles that of other R proteins after effector recognition (Dodds et al., 2006; Takken and Goverse, 2012). In mouse, the NLR NLRC4 is normally in an auto-inhibited state, that is presumably relieved upon ligand interaction with the specificity-determining NAIP and subsequent interactions between NLRC4 and NAIP/ligand complex (Kofoed and Vance, 2011; Tenthorey et al., 2014). In contrast, RPS4 and RRS1 are constitutively associated (Williams et al., 2014).

Mutations in several *Arabidopsis* WRKY genes, notably WRKY33, are associated with reduced pathogen resistance (Birkenbihl et al., 2012). We propose both AvrRps4 and PopP2 evolved to modulate or block WRKY protein function. WRKY41 interaction with AvrRps4 was first reported in yeast 2-hybrid screens (Mukhtar et al., 2011). In turn, effector interference with WRKY proteins led to selection for a resistance complex, RPS4/RRS1, which detects effectors that target WRKY domains, and we hypothesize that the *C. higginsianum* fungal effector recognized by RPS4/RRS1-R also binds WRKY domains.

Other *R* gene pairs exist in which one of the two members encode additional protein domains. Notably, in the *RGA4* and

RGA5 gene pair in rice, *RGA5* encodes a C-terminal extension with an RATX1 domain, which interacts with recognized effectors (Césari et al., 2013, Césari et al., 2014). However, these effectors have not been reported to target other RATX1-domain proteins involved in or required for disease resistance. These protein architectures suggest that RPS4/RRS1 provides an archetype for 2-component immune complexes in which one of the two NB-LRR proteins has integrated a decoy domain that enables the plant to detect effectors that interact with important immunity components that carry this domain. The presence of protein domain fusions in many NB-LRR immune receptors suggests *R* gene pairs with integrated decoy (Césari et al., 2014) domains may be widespread in angiosperms. We suggest that such protein domains in other NB-LRR proteins are effector targets, and such NB-LRR domain fusions are likely to require a partner NB-LRR protein for defense activation.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Nicotiana benthamiana (*Nb*) and *N. tabacum* (*Nt*) "Petit Gerard" plants were grown in long days (16 hr light/8 hr dark) at 24°C. *Arabidopsis* plants were grown in short days (10 hr light/14 hr dark) at 21°C or 28°C.

Plasmid Constructions

Golden Gate cloning and site-directed mutagenesis methods are provided in the Supplemental Experimental Procedures.

Agrobacterium-Mediated Transient Transformation of *N. benthamiana* and *N. tabacum*

Agrobacterium strains carrying various constructs were grown in liquid LB-medium supplemented with antibiotic for 24 hr. Cells were harvested by centrifugation, washed in 5 ml of 10 mM $MgCl_2$, and re-suspended at OD_{600} 0.5 in infiltration medium (10 mM $MgCl_2$, 10 mM MES [pH 5.6]). *Nt* was infiltrated for HR assays and *Nb* for protein expression at 4–5 weeks old.

Protein Extraction, Immunoprecipitation, and Immunoblotting

Protein samples were immunoprecipitated from *Nb* 48 hr after *Agrobacterium* infiltration. Purified proteins were separated by SDS-PAGE and analyzed by immunoblotting using anti-FLAG M2- (Sigma) or anti-HA-HRP (Roche) or GFP-HRP (Santa Cruz) conjugated antibodies. For further details see the Supplemental Experimental Procedures.

Gel Mobility Shift Assay

Detailed protocols are in the Supplemental Experimental Procedures. RRS1-R/S-HF, RPS4-HA, PopP2-/PopP2-C321A-GFP, AvrRps4/AvrRps4-E187A-GFP, GFP, and GFP-FLAG were expressed and extracted, IP purified from *Nb* after *Agrobacterium* infiltration. Synthetic W-box DNA (W-box: 5′-CGTTGACCGTTGACCGAGTTGACTTTTTA-3′), or a mutant form (mW-box: 5′-CGTaGACgGTaGACgGAGTaGACgTTTTA-3′), were 5′-labeled and incubated with freshly prepared protein. Samples were separated on a 4% native gel in 0.5× TBE buffer, 100 Volt for 45 min, the gel was dried on 3MM paper and exposed.

Mass Spectrometry Analysis

Detailed protocols are in the Supplemental Experimental Procedures. Samples for liquid chromatography-mass spectrometry (LC-MS) analysis were excised from SDS-PAGE gels, destained, reduced, and alkylated, and digested with trypsin. Peptides were analyzed on a hybrid mass spectrometer LTQ-Orbitrap XL (ThermoFisher Scientific) and a nanoflow-UHPLC system (nanoAcquity, Waters) and masses were searched on Mascot server v.2.4.1 (Matrix Science) against the TAIR (version 10) database to identify acetylated peptides. Selected reaction monitoring (SRM) of acetylated peptides and non-modified control peptides by triple quadrupole MS was performed as described in Kadota et al. (2014) using nano-spray LC ESI and a TQ-S MS (Waters). At least one replicate injection was performed per experiment and each experiment was repeated at least three times. The resultant TQ-S files were analyzed in Skyline (see Supplemental Experimental Procedures).

Pseudomonas fluorescens Pf0-1 and *P. syringae* pv. tomato DC3000 Assays

PopP2 expression and delivery from *P. fluorescens* Pf0-1 with a type III secretion system [Pf0-1 (T3SS)], was described (Sohn et al., 2014). For infiltration into *Arabidopsis* leaves, Pf0-1 (T3SS) and *P. syringae* pv. *tomato* DC3000 carrying the pEDV6 AvrRps4 strains grown on King's B agar plate with antibiotics (Chloramphenicol 30 μg/ml, Tetracycline 5 μg/ml or gentamicin 20 μg/ml) were harvested and prepared (A_{600} = 0.2) for inoculation as described (Sohn et al., 2014).

Confocal Microscopy

Methods for confocal microscopy imaging, after *Nb* leaf infiltration were performed as described (Sohn et al., 2012).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.04.024.

AUTHOR CONTRIBUTIONS

P.F.S., K.H.S., Y.M., C.S., Z.D., S.U.H., and J.D.G.J. designed the research. P.F.S., Z.D., S.U.H., Y.M., C.S., and V.C. performed the research. J.S., G.R., L.W., and S.B.S. contributed materials or analytic tools. P.F.S., Z.D., Y.M., J.S., P.D., F.L.H.M., S.U.H., and J.D.G.J. analyzed data. P.F.S., Z.D., Y.M., and J.D.G.J. wrote the paper.

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